An Infrared Spectroscopic Study of the Interactions of Carbohydrates with Dried Proteins†

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ABSTRACT: Fourier-transform infrared spectroscopy was used to characterize the interaction of stabilizing carbohydrates with dried proteins. Freeze-drying of trehalose, lactose, and *myo*-inositol with lysozyme resulted in substantial alterations of the infrared spectra of the dried carbohydrates. In the fingerprint region (900–1500 cm^{-1}), there were large shifts in the frequencies of bands, a decrease in absorbance, and a loss of band splitting. These effects mimic those of water on hydrated trehalose. Bands assigned to hydroxyl stretching modes (around 3350 cm^{-1}) were decreased in intensity and shifted to higher frequencies in the presence of the protein. In complementary experiments, it was found that dehydration-induced shifts in the positions of amide I and amide II bands for lysozyme could be partially and fully reversed, respectively, when the protein was freeze-dried in the presence of either trehalose or lactose. In addition, the carboxylate band, which was not detectable in the protein dried without the sugar, was apparent when these sugars were present. *myo*-Inositol was less effective at shifting the amide bands, and the carboxylate band was not detected in the presence of this carbohydrate. Also tested was the concentration dependency of the carbohydrates' influence on the position of the amide II band for dried lysozyme. The results showed that the ability of a given concentration of a carbohydrate to shift this band back toward the position noted with the hydrated protein coincided, at least in the extreme cases, with the capacity of that same level of carbohydrate to preserve the activity of rabbit skeletal muscle phosphofructokinase during freeze-drying. Taken together, these results suggest not only that hydrogen bonding occurs between dried proteins and carbohydrates but also that carbohydrate binding is requisite for carbohydrate-induced stabilization of proteins during freeze-drying and rehydration.

Addition of stabilizing solutes to labile enzymes is a common means of protecting such proteins during preparation and storage. A wide variety of solutes, including sugars, polyols, amino acids, methylamines, and lyotropic salts, are effective at minimizing protein denaturation in the face of stresses (e.g., high temperature) imposed in aqueous systems (Gekko & Morikawa, 1981; Lee & Timasheff, 1981; Timasheff, 1982; Arakawa & Timasheff, 1982a,b, 1983, 1985; Low, 1985). These same solutes have the capacity to protect even extremely labile enzymes, such as phosphofructokinase and lactate dehydrogenase, during freeze–thawing (Carpenter et al., 1986; Carpenter & Crowe, 1988a). In contrast, we have found that only certain carbohydrates (e.g., disaccharides) can preserve phosphofructokinase activity during either freeze-drying or air-drying, indicating that the mechanism by which stabilizing solutes protect dried proteins may be fundamentally different from that seen in solution or during freeze–thawing (Carpenter et al., 1987a,b; Carpenter & Crowe, 1988b). In the present study, we have used Fourier-transform infrared spectroscopy to investigate the mechanism of the interaction of stabilizing carbohydrates with dried proteins.

Timasheff, Arakawa, and their colleagues have shown that all of the classes of solutes mentioned above protect proteins in aqueous solution because they are preferentially excluded from the domain of the protein and the protein is preferentially hydrated, thus making it thermodynamically unfavorable for the protein to unfold [cf. Timasheff (1982)]. Recently, we have demonstrated that cryoprotection of proteins by these solutes can be explained by the same mechanism (Carpenter & Crowe, 1988a). However, this generalization is not applicable to dried systems since many cryoprotectants do not confer stability to dried proteins (Carpenter et al., 1987a,b; Carpenter & Crowe, 1988b). In addition, the thermodynamic arguments that are needed to explain protein stabilization by preferentially excluded solutes are not applicable when water is removed from the system. In a previous report, we suggested that certain carbohydrates might protect dried proteins because these solutes bind to the dried protein, thus serving as a "water substitute", when the hydration shell of the protein is removed (Carpenter & Crowe, 1988b). In the following account, we provide evidence not only that hydrogen bonding occurs between stabilizing carbohydrates and dried proteins but also that solute binding is requisite for labile proteins to be preserved during drying.

Materials and Methods

Materials. Hen egg white lysozyme (HCl) was purchased from Boehringer-Mannheim and bovine serum albumin (BSA)[1] from Sigma. These proteins were obtained as lyophilized powders and used without further purification. Rabbit skeletal muscle phosphofructokinase from Sigma was purified following the protocol described by Carpenter et al. (1986). Deuterium oxide (99.8 atom % D) was obtained from Sigma. All carbohydrates were purchased from Pfanstiehl Laboratories.

Sample Preparation and Infrared Spectroscopy. Infrared spectra were taken with a Perkin-Elmer Fourier-Transform

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[1] Abbreviation: BSA, bovine serum albumin.

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Infrared Spectrometer (Model 1700), and data were acquired by using a Perkin-Elmer 7500 data station. All spectra were smoothed using the Savitzky–Golay function with 13 points. For experiments testing the influence of protein on the infrared spectrum of dried carbohydrates, solutions of carbohydrates alone and carbohydrate/protein mixtures were prepared in distilled and deionized water. Samples (0.5 mL) were frozen in liquid nitrogen and then lyophilized (15 mTorr) for at least 48 h on a VirTis lyophilizer. Approximately 5–30 mg of a dried sample was weighed to the nearest 0.01 mg, mixed with 495 mg of dry KBr, and ground to a fine powder with an agate mortar and pestle. Approximately 100 mg of the resulting powder (weighed to the nearest 0.01 mg) was placed in a Perkin-Elmer casting die, to which a vacuum pump was attached, and pressed into a disk. Differences in the weights of carbohydrate between disks were normalized on the data station after the infrared spectra were recorded. In this fashion, the influence of protein on the intensity of the carbohydrates' infrared spectra as well as shifts in band frequency could be evaluated.

In experiments testing the influence of carbohydrates on the infrared spectra of dried lysozyme, all solutions were prepared in deuterium oxide. Samples were freeze-dried and ground with KBr as above. The resulting powder was placed in the casting die, which was then placed in a specially modified desiccator connected to the lyophilizer. In this arrangement, vacuum could be applied directly to the vacuum port of the die (via an in-line valve) as well as to the desiccator. After at least 24 h at 15 mTorr, the vacuum to the desiccator was released, and the in-line valve to the casting die was closed, prior to disconnecting the die from the lyophilizer. The die was then connected to a vacuum pump, the in-line valve was opened, and the powder in the die was pressed into a disk. After pressing, the in-line valve was closed (while vacuum was still being applied to the die), and the die was tranferred to a dry box purged with dry nitrogen gas. In the dry box, the KBr disk was removed from the die and mounted between two BaF windows, within an "O" ring seal. With this sealed system, infrared spectra could be taken without exposure of the disk to moisture in the air.

To obtain the infrared spectrum of fully hydrated lysozyme, a solution of 200 mg/mL was prepared in deuterium oxide. Spectra were acquired from a thin film of this preparation mounted between two BaF windows. For comparison of spectra between hydrated and dried protein samples (and samples dried with carbohydrates), the Abex routine of the Perkin-Elmer CDS-3 software (which expands the spectra so that relative heights of the bands are maintained while the highest band is expanded to a predetermined absorbance) was used.

Freeze–Thawing and Freeze-Drying of Phosphofructokinase. Prior to each experiment, phosphofructokinase was dialyzed (4 °C) for several hours against 10 mM potassium phosphate buffer (pH 8.0 at 23 °C) containing 5 mM dithiothreitol. An aliquot of the stock enzyme was then added to the appropriate solution of carbohydrate (prepared in the above buffer) to give the desired concentration of carbohydrate. The final phosphofructokinase concentration was 50 μg/mL in experiments with trehalose and lactose and 150 μg/mL in experiments with inositol. After the initial catalytic activity of a given sample was measured (Carpenter et al., 1986), a 75-μL aliquot was transferred to a polypropylene Eppendorf test tube and frozen in liquid nitrogen for 30 s. Samples were thawed at room temperature, mixed, and immediately assayed for residual catalytic activity. Another 75-μL aliquot was frozen in liquid nitrogen and then placed on a VirTis lyophilizer overnight at 15 mTorr. Freeze-dried samples were rehydrated in 5 mM dithiothreitol (pH 7.8 at 23 °C), and residual phosphofructokinase activity was assayed.

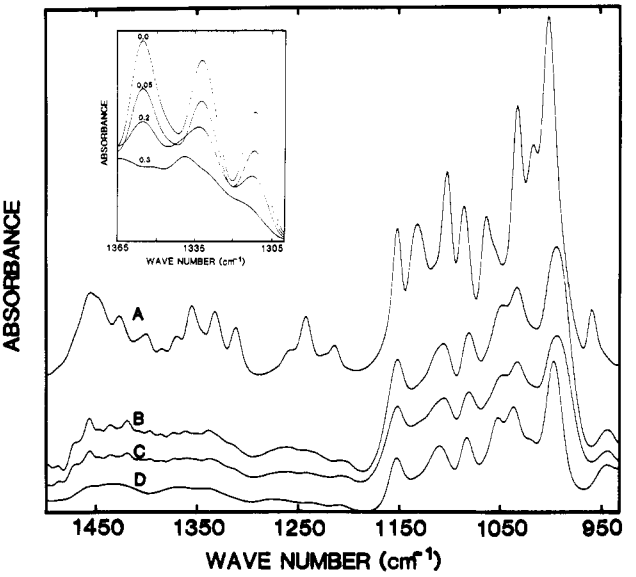

FIGURE 1: Infrared spectra in the fingerprint region for trehalose freeze-dried alone (spectrum A), in the presence of 0.3 g of lysozyme/g of trehalose (spectrum B), and in the presence of 0.1 g of BSA/g of trehalose (spectrum C) and for hydrated trehalose (spectrum D). The spectra for the dried samples have been corrected for differences in the amounts of trehalose present in the samples and can be compared quantitatively as well as qualitatively. The spectrum for the hydrated sugar has been normalized, relative to the band at approximately 1000 cm^{-1} (which was previously normalized to the corresponding band in the bovine serum albumin/sugar spectrum), by using the Abex routine (see Materials and Methods); therefore, only qualitative comparison can be made between this spectrum and the spectra for the dried samples. The spectra are offset from each other for clarity. Inset: Infrared spectra in the region tentatively assigned to hydroxyl bending modes for trehalose freeze-dried alone and with the indicated amounts of lysozyme. Values given represent the grams of lysozyme per gram of trehalose. The spectra have been corrected for differences in the amounts of trehalose present in the samples and can be compared quantitatively.

RESULTS

Influence of Proteins on the Infrared Spectra of Dried Carbohydrates. Infrared spectra of crystalline sugars are complex, with many unassigned bands (Tipson & Parker, 1980). Nevertheless, alterations in individual vibrational modes and changes in coupled vibrations can be used to assess hydrogen bonding by sugars (Mitchell & Higgins, 1965; Mitchell, 1968a,b; Kogan et al., 1973; Umemura et al., 1979; Tipson & Parker, 1980; Szarek et al., 1984). Therefore, in order to investigate the possibility that there is hydrogen bonding between sugars and proteins that have been freeze-dried together, we determined the influence of proteins on the infrared spectrum of trehalose.

Figure 1 compares the fingerprint region of the infrared spectrum for trehalose dried alone with that for trehalose dried in the presence of 0.3 g of lysozyme/g of sugar. The presence of the protein leads to a pronounced decrease in absorbance in the entire region, major shifts in band position, and a loss of band splitting. These effects are even more pronounced when trehalose is freeze-dried in the presence of 0.1 g of BSA/g of sugar (Figure 1). The significance of the influence of proteins on the vibrational spectrum of the dried sugar can best be appreciated when compared to the effects of water on the spectrum on hydrated trehalose (Figure 1). A typical spectrum for trehalose dried in the presence of BSA is re-

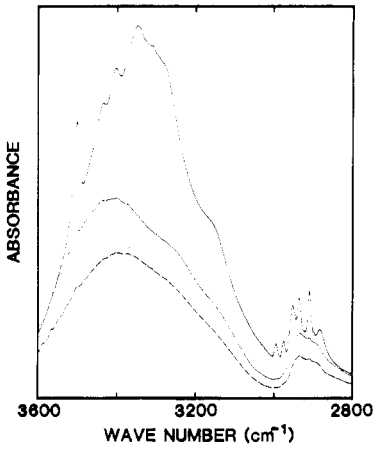

FIGURE 2: Infrared spectra in the hydroxyl stretching region for trehalose freeze-dried alone (solid line) and in the presence of 0.3 g of lysozyme/g of trehalose (dotted line) or 0.1 g of bovine serum albumin/g of trehalose. (dashed line). The spectra have been corrected for differences in the amounts of trehalose present in the samples. The contributions of the proteins to the absorbance in this region have been subtracted from the latter two spectra.

markably similar to that for hydrated trehalose, while both are very different from a spectrum of crystalline trehalose. However, the bands seen for the hydrated trehalose are at a slightly higher frequency than those for the dried sugar/protein samples.

The protein-induced spectral changes in the dried sugar can be titrated by freeze-drying the sugar with increasing amounts of either protein. This influence can be seen most clearly in the region from 1365 to 1300 cm^{-1} (Figure 1), which contains bands assigned to plane-bending hydroxyl modes in sugars (Mitchell, 1968b; Vasko et al., 1972; Cael et al., 1974). In the presence of increasing amounts of lysozyme, there is a progressive decrease in the absorbance in the three bands and a shift to a higher frequency. Similar titrations on the sugar spectrum can be accomplished with BSA (data not shown).

Lysozyme and BSA also have pronounced effects on the hydroxyl stretching modes and the CH_2 stretching modes [cf. Tipson and Parker (1980)] of dried trehalose (Figure 2). With either protein, there is a fusion of the six sharp CH_2 stretching bands (around 2850 cm^{-1}) into one broad band. In addition, there is a decrease in the absorbance and an increase in the frequency of the broad hydroxyl stretching band centered around 3350 cm^{-1}. Also, the many sharp shoulders seen in this band in the absence of protein are lost. As was noted above for the fingerprint region, these protein-induced effects can be titrated by freeze-drying trehalose with increasing amounts of either protein (data not shown).

It might be that the influence of the proteins on the sugar is not due to the interaction of the protein with the sugar during freeze-drying but rather due to associations occurring during the formation of the KBr disks. To assure that this is not the case, 0.3 mg of dried lysozyme or dried BSA was combined with 1 mg of dried trehalose. These preparations were ground with dried KBr and pressed into disks. Except for the presence of bands assigned to the proteins, the spectra of these samples are indistinguishable from those seen for dried trehalose alone (data not shown).

We next wanted to determine if, as expected, the effects of proteins on dried trehalose could be generally seen with other carbohydrates. In Figure 3A, it can be seen that the spectrum of lactose freeze-dried in the presence of 0.3 g of lysozyme/g of sugar was also greatly altered relative to the spectrum seen with the sugar alone. Similar results were obtained with

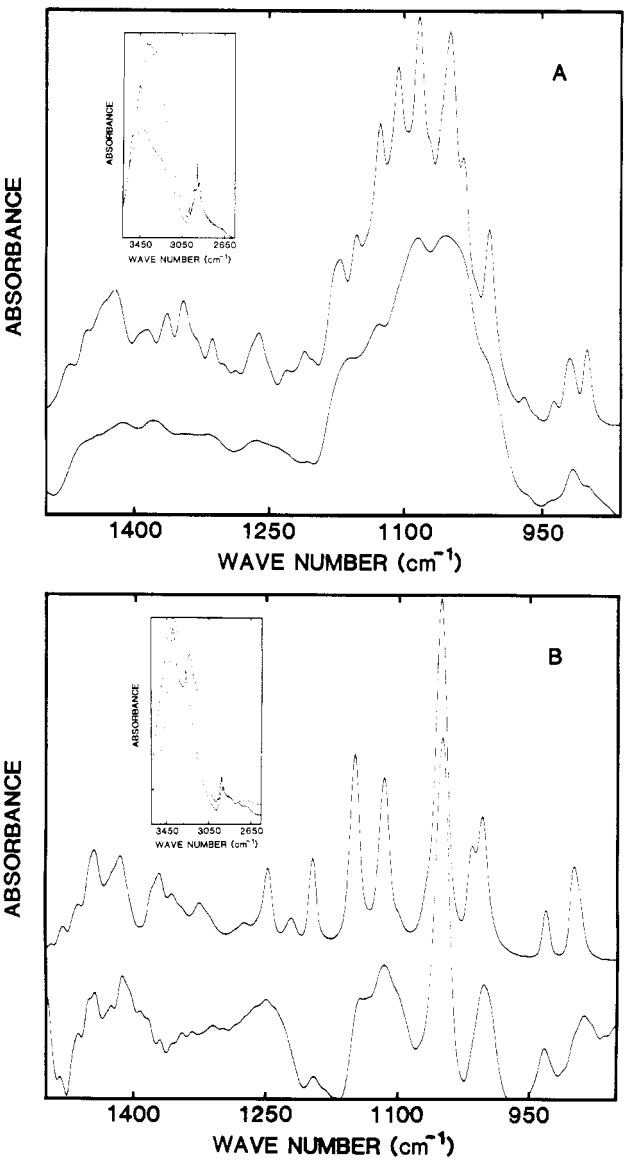

FIGURE 3: (A) Infrared spectra for lactose freeze-dried alone (upper spectrum) and in the presence of 0.3 g of lysozyme/g of lactose (lower spectrum). Inset: Infrared spectra in the hydroxyl stretching region for lactose in the presence (dotted line) and absence of lysozyme (solid line). The contribution of lysozyme to the absorbance in this region has been subtracted from the spectra. (B) Infrared spectra for inositol freeze-dried alone (upper spectrum) and in the presence of 3.0 g of lysozyme/g of inositol (lower spectrum). Inset: Infrared spectra in the hydroxyl stretching region for inositol in the presence (dotted line) and absence of lysozyme (solid line). The contribution of lysozyme to the absorbance in this region has been subtracted from the spectra. The spectra in panels A and B have been corrected for differences in the amounts of lactose or inositol, respectively, present in the samples and are offset for clarity.

myo-inositol (Figure 3B), but almost an order of magnitude more lysozyme was required to alter the spectrum to a comparable degree. In fact, essentially no change was detectable in the *myo*-inositol spectrum unless greater than 0.2 g of lysozyme/g of *myo*-inositol was present (data not shown). As was the case with trehalose, the protein-induced shifts in either the lactose or the *myo*-inositol spectra could be titrated (data not shown).

Influence of Carbohydrates on the Infrared Spectra of Dried Lysozyme. When lysozyme is dried, there is an increase in the frequency of the amide I band from 1652.5 cm^{-1}, seen for the fully hydrated protein, to 1659 cm^{-1} (Figure 4). The amide II band is broadened and shifts from about 1543 cm^{-1} to almost 1530 cm^{-1} in the dried protein. In addition, the band

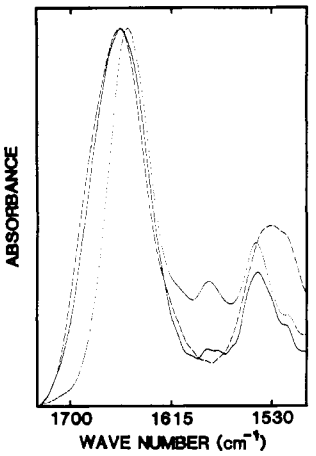

FIGURE 4: Amide band region for hydrated lysozyme (dotted line), lysozyme freeze-dried alone (dashed line), and lysozyme (20 mg/mL) freeze-dried in the presence of 100 mg/mL trehalose (solid line). All spectra have been normalized by using the Abex routine (see Materials and Methods).

assigned to carboxylate in the hydrated protein (Careri et al., 1979) at 1583 cm^{-1} is not detectable with the dried protein. These dehydration-induced spectral changes are well documented and have been shown to vary in degree with the hydration level of lysozyme (Careri et al., 1979, 1980; Poole & Finney, 1982, 1984).

When 20 mg/mL lysozyme is freeze-dried in the presence of 100 mg/mL trehalose, the amide I band of the dried protein is shifted back to 1658.1 cm^{-1} (Figure 4). There also is the appearance of a band corresponding to that for carboxylate at 1583 cm^{-1}. The most dramatic effect of the sugar is to shift the amide II band back to 1542 cm^{-1}, almost the identical position noted for the hydrated protein. In addition, the band shape is essentially the same as that seen for the hydrated protein. To ascertain that these sugar-induced alterations in the spectrum of dried lysozyme were due to sugar binding to the protein during freeze-drying and not due to interaction occurring during the formation of the KBr disk, 1 mg of dried lysozyme was combined with 5 mg of dried trehalose, ground with KBr, and pressed into a disk. The lysozyme spectrum in this preparation was identical with that seen with the protein alone (data not shown).

The spectral characteristic of dried lysozyme that is most sensitive to the presence of added trehalose is the position of the amide II band. Therefore, we used this parameter as a means to characterize the effect of varying the amount of sugar on the vibrational spectrum of dried lysozyme. These experiments led to some surprising results. As can be seen in Figure 5A, as expected, there is an increase in the amide II frequency as the sugar concentration is increased up to 100 mg/mL. With 100–200 mg/mL trehalose, the amide II band is centered at about 1542 cm^{-1}. However, when the sugar concentration is greater than 200 mg/mL, there is a progressive decrease in the frequency of the amide II band, such that with 400 mg/mL trehalose, amide II is shifted back to 1531 cm^{-1} (Figure 5A). A concentration of 400 mg/mL is very near the limit of solubility of trehalose at room temperature. Interestingly, in the lysozyme samples freeze-dried with this level of sugar, the bands in the trehalose fingerprint region show a high degree of splitting, a characteristic feature of dried, crystalline trehalose (data not shown). The occurrence of such crystallization during sublimation could decrease the availability of the sugar for forming hydrogen bonds with the protein. In contrast, with the lower initial concentrations of sugar, which lead to increased frequency of the amide II band, band splitting in the trehalose fingerprint region is absent.

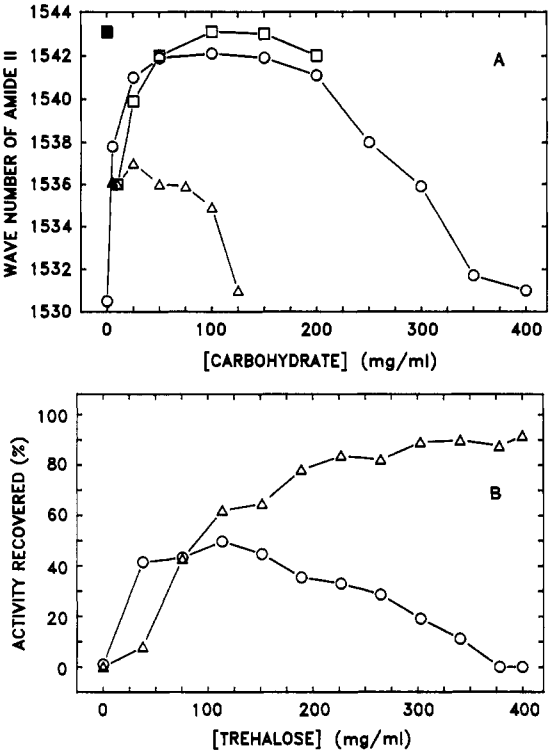

FIGURE 5: (A) Effects of trehalose (○), lactose (□), and inositol (△) on the wavenumber for the amide II band of dried lysozyme. Lysozyme (20 mg/mL) was freeze-dried in the presence of the indicated amounts of the carbohydrates. The solid square represents the wavenumber for the amide II band of hydrated lysozyme. (B) Comparison of the percentage of phosphofructokinase activity recovered after freeze-thawing (△) or freeze-drying and rehydration (○) in the presence of trehalose.

On the basis of these results, which suggest decreased interaction between the sugar and protein when high initial concentrations of the sugar are used, we investigated the ability of trehalose to preserve the activity of a labile protein (phosphofructokinase) during freeze-drying in the presence of a broad range of initial sugar concentrations. Freeze-drying of phosphofructokinase leads to complete, irreversible inactivation (Figure 5B; Carpenter et al., 1986, 1987a). The greatest degree of protection is noted with intermediate amounts of sugar (Figure 5B). With sugar concentrations greater than 150 mg/mL, there is a decrease in activity recovered, such that no stabilization is noted with 400 mg/mL trehalose. These results indicate that whenever trehalose is at a concentration that does not influence the frequency of the amide II band for dried lysozyme, this sugar concentration is also ineffective at preserving dried, labile proteins.

To test this suggestion further, we determined the influence of lactose and *myo*-inositol on the infrared spectrum of dried lysozyme (Figure 6). With 100 mg/mL lactose, there are similar effects to those seen with trehalose. The amide I band is shifted back to 1656.7 cm^{-1}, the carboxylate band at 1583 cm^{-1} is detectable, and the amide II band is shifted to 1543 cm^{-1} (Figure 6A). However, the concentration dependency of these lactose-induced effects is different than the results noted with trehalose. Even when lactose is present at an initial concentration very near its limit of solubility at room temperature (i.e., 225 mg/mL), there is little loss in the sugar's capacity to shift amide II to higher frequencies (Figure 5A). However, the ability of lactose to stabilize dried phosphofructokinase also does not diminish at the highest sugar con-

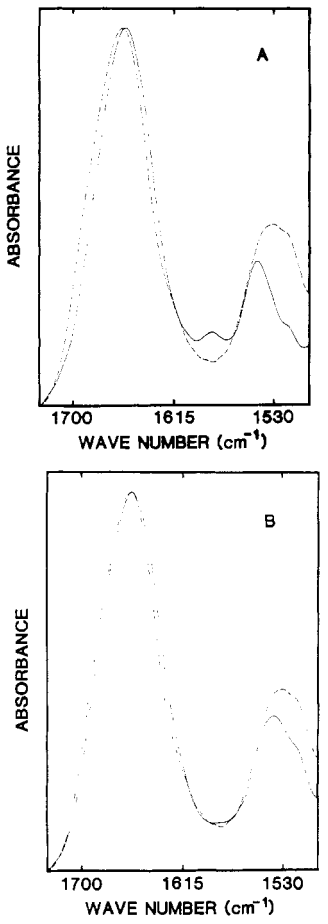

FIGURE 6: (A) Amide band region for dried lysozyme (dashed line) and lysozyme (20 mg/mL) dried in the presence of 100 mg/mL lactose (solid line). (B) Amide band region for dried lysozyme (dashed line) and lysozyme (20 mg/mL) dried in the presence of 25 mg/mL inositol (solid line). All spectra have been normalized by using the Abex routine (see Materials and Methods).

centrations tested (Figure 7A).

Earlier studies from our laboratory have shown that, in comparison to the disaccharides, *myo*-inositol affords minimal protection to labile proteins (Carpenter et al., 1987a,b). Thus, as might be expected, even the most effective concentration of *myo*-inositol had much less influence on the infrared spectrum of dried lysozyme than did the disaccharides (Figure 6B). The maximum shift of the amide II band is to 1537 cm^{-1}, in the presence of an initial *myo*-inositol concentration of 25 mg/mL. As was the case with trehalose, at a concentration nearing its limits of solubility at room temperature (i.e., 125 mg/mL), *myo*-inositol had almost no influence on the amide II frequency of dried lysozyme (Figure 5A).

To be able to detect stabilization of phosphofructokinase by *myo*-inositol, it was necessary to increase the concentration of the enzyme from 50 μg/mL (the concentration used in experiments with trehalose and lactose) to 150 μg/mL. In many instances, it has been found that increasing protein concentration leads to greater enzyme stability during freeze–thawing or freeze-drying [cf. Carpenter and Crowe (1988a,b)]. Even under these conditions, *myo*-inositol provides less protection to dried phosphofructokinase than does lactose or trehalose (Figure 7B). However, the concentration dependency of *myo*-inositol-induced preservation of the freeze-dried enzyme is similar to that seen with trehalose (Figures 5B and 7B). At *myo*-inositol concentrations above 50 mg/mL, there is decreased protection, and no enzyme activity is detectable in samples freeze-dried with 125 mg/mL *myo*-inositol (Figure 7B). These results support the earlier suggestion that,

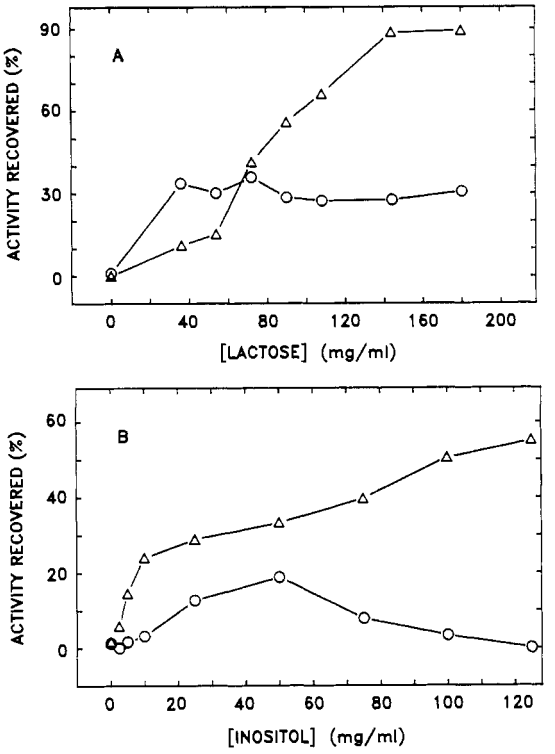

FIGURE 7: Comparison of the percentage of phosphofructokinase activity recovered after freeze–thawing (Δ) or freeze-drying and rehydration (O) in the presence of (A) lactose and (B) inositol.

at least in the extreme cases, the failure of a given concentration of carbohydrate to alter the infrared spectrum of dried lysozyme coincides with the inability of that concentration of carbohydrate to preserve labile enzymes.

Finally, in contrast to the results noted for freeze-drying of phosphofructokinase, during freeze–thawing the presence of increasing concentrations of carbohydrates leads to increased recovery of activity (Figures 5B and 7). These results are as expected since, as explained in the introduction, it is known that cryoprotection is due to the preferential exclusion of the stabilizing solute from the surface of the protein (Carpenter & Crowe, 1988a). Even at very high concentrations of sugar, there should still be preferential exclusion (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982) and hence cryoprotection.

Discussion

Infrared spectroscopy provides a convenient and a powerful means to monitor hydrogen bonding between two molecular species in the solid state. In the following paragraphs, we discuss how alterations in the vibrational spectra of carbohydrates dried in the presence of proteins and the complementary influence of carbohydrates on dried protein spectra are indicative of sugar–protein hydrogen bonding. We then relate the importance of this interaction to the stabilization of dried proteins by carbohydrates.

In studies on hydrogen bonding by carbohydrates, the feature of the infrared spectrum most often monitored is the hydroxyl stretching region, found from 3650 to 3100 cm^{-1} (Mitchell & Higgins, 1965; Mitchell, 1968a,b; Kogan et al., 1973; Umemura et al., 1979; Szarek et al., 1984). The large widths of these bands and the fine structure in this region are thought to arise from coupled vibrations rather than from the separate vibrations of individual groups (Mitchell, 1968a). Therefore, it is not possible to assign individual features to specific hydroxyl groups. However, the position of the broad band is indicative of the type of hydrogen bonding in which the constituent hydroxyl groups are involved. For example,

the OH stretching bands for dried trehalose, lactose, and *myo*-inositol are centered around 3350 cm^{-1}. This position indicates that there is intermolecular hydrogen bonding between the carbohydrate molecules (Mitchell & Higgins, 1965; Mitchell, 1968a,b; Kogan et al., 1973; Parker, 1971; Tipson & Parker, 1980).

When *myo*-inositol is dried in the presence of lysozyme, there is a fusion of the bimodal peak into a single peak and an overall shift of the hydroxyl band to higher frequency (Figure 3B). With lactose or trehalose, the hydroxyl stretching band is decreased in intensity and shifted to a higher frequency (Figures 2 and 3A). Similar alterations in the hydroxyl stretching absorbance have been measured for carbohydrates in dilute solution and in nonaqueous solvents, in which intermolecular hydrogen bonds cannot form (Mitchell & Higgins, 1965). This is consistent with the observation that, in general, disruption of hydrogen bonds leads to an increase in the frequency and a decrease in the intensity of hydroxyl stretching bands (Hadzi & Bratos, 1976; Bellamy, 1980).

Thus, our results indicate that when carbohydrates are dried in the presence of proteins, the capacity of carbohydrate molecules to form intermolecular hydrogen bonds between themselves is diminished. This appears to result from formation of hydrogen bonds between the carbohydrate molecules and the protein. In the crystalline sugar, the sugar molecules should be able to form more intermolecular hydrogen bonds per mole of sugar than would sugar molecules that are hydrogen bonded to dried protein. Therefore, the influence of sugar molecules, which hydrogen bond to the dried protein, on the coupled vibrations comprising the hydroxyl stretching band would not be sufficient to offset the effect due to the loss of hydrogen bonding between sugar molecules. This could account for the fact that there is still an increase in frequency and a decrease in intensity of the hydroxyl stretching band, even though some hydroxyls in the sugar are hydrogen bonded to the protein.

Further support for this contention comes from examination of the fingerprint region of trehalose. The loss of band splitting and the shifts in bands seen when the sugar is dried with proteins are almost identical with that seen when trehalose is dried with phospholipids (Crowe et al., 1984a,b, 1985). These phospholipid-induced alterations of the sugar's infrared spectrum, in particular the loss of bands in the region of 1300–1365 cm^{-1} that are tentatively assigned to in-plane hydroxyl deformations (Mitchell, 1968b; Vasko et al., 1972; Cael et al., 1974), have been interpreted to be due to the hydrogen bonding between hydroxyls in the sugar and the phospholipid headgroups (Crowe et al., 1984a,b, 1985). This conclusion has recently gained further support from several independent lines of evidence including results from ^{31}P NMR, studies demonstrating competition of sugars with europium for binding to the phosphate headgroup, and molecular modeling of sugar–phospholipid interactions [reviewed in Crowe et al. (1987, 1988)].

Perhaps even more compelling are our results comparing the effects of water on hydrated sugar to those of proteins on the dried sugar (Figure 1). The spectral changes are strikingly comparable, indicating that the protein affects the dried sugar similarly to the effects of water on hydrated trehalose. That is, protein and water minimize direct sugar–sugar interactions since they, themselves, form hydrogen bonds with the sugar molecules. The fact that, overall, the frequency of bands in the fingerprint region is somewhat lower in the dried preparations than in the hydrated state does not contradict this conclusion. This observation is most likely due to the limited mobility and kinetic energy in the dried state compared to hydrated systems.

It is implicit in the conclusion that proteins serve as water substitutes for dried carbohydrates, by satisfying the hydrogen-bonding requirements for the sugar, that the converse must also be true. If the hydrogen-bonding requirements of the protein [cf. Edsall and McKenzie (1983)] are not satisfied, then there is a drastic alteration in the infrared spectrum of the protein (Figure 4). Both the direction and the degree of the shifts in the position of amide I and II bands that we noted for the dried protein are comparable to those seen by earlier researchers with lysozyme and other proteins (Ruegg & Hani, 1975; Careri et al., 1979, 1980; Poole & Finney, 1982, 1984). There has been some controversy in the literature concerning the cause for these large dehydration-induced shifts. Careri and his colleagues have suggested that these changes are entirely due to the removal of water from polar groups (Careri et al., 1979, 1980) whereas Poole and Finney (1983) contend that alterations in protein conformation may contribute to the change in the vibrational spectrum of dried lysozyme (Finney & Poole, 1984). For our purposes, only the fact that these alterations can be induced by dehydration is of concern since we wanted simply to compare the influence of water on the hydrated protein to that of carbohydrates on the dried protein.

As expected, we find that dehydration-induced alterations in the vibrational spectrum of lysozyme could be reversed, at least in part, if the protein is dried in the presence of carbohydrates (Figures 4–6). The fact that the dehydration-induced alterations in the lysozyme spectrum are not all fully reversed (e.g., the shift in amide I) by the carbohydrates may be due to steric limitations in the sugar's access to the polar groups on the protein. Also, the different degrees of sugar-induced changes in amide I versus amide II are not that surprising since these two bands are characterized by vibrations from different groups and provide different information about the protein. Amide I is mainly carbonyl stretch, whereas amide II is composed of bending and stretching modes, primarily N–H bend (Wharton, 1986). Also, the former is much more sensitive to changes in the conformation and structure of the protein than the latter (Gendreau, 1986). Thus, our results indicate that carbohydrates appear to be more effective at reversing changes in the infrared spectrum of lysozyme that derive directly from removal of water (shift of amide II to a lower frequency and loss of the carboxylate band at 1583 cm^{-1}) than at attenuating alterations due to putative conformational changes (shift of amide I to higher frequency). However, as noted above, our experiments were not designed to differentiate between the relative contributions of dehydration and conformational changes to the alterations in the infrared spectrum of lysozyme seen after drying. Therefore, we think the most appropriate conclusion to draw from our results is that carbohydrates can serve to satisfy, to at least a limited degree, the hydrogen-bonding requirements of the polar groups in the dried protein, and thus serve as a water substitute for dried proteins.

By comparing the concentration dependency of carbohydrate-induced shifts in the amide II band of dried lysozyme with that for preservation of phosphofructokinase (Figures 5 and 7), one can draw conclusions concerning the role of carbohydrate binding in the stabilization of dried proteins. It appears that whenever a given concentration of carbohydrate does not alter the spectrum of dried lysozyme, that same initial level of carbohydrate does not stabilize dried phosphofructokinase (Figures 5 and 7). Thus, we conclude not only that our results indicate that hydrogen bonding can occur between

dried proteins and carbohydrates but also that such binding is mandatory for the carbohydrate to confer stability to dried proteins.

During drying, the major stress that must be overcome is the removal of the protein's hydration shell, which, for at least some labile enzymes, can result in irreversible inactivation upon rehydration. Replacement of the lost water, by the hydrogen bonding of carbohydrate to the protein's polar groups, appears to be responsible for preventing dehydration-induced inactivation. The mechanism by which this interaction results in enzyme preservation is not known at the present time, but the data and interpretations presented here should serve as a guide for future formulations of the molecular and/or thermodynamic mechanisms.

Registry No. Lysozyme, 9001-63-2; trehalose, 99-20-7; lactose, 63-42-3; inositol, 87-89-8; phosphofructokinase, 9001-80-3.

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